

A Network of Transverse and Longitudinal Intermediate Filaments Is Associated with Sarcomeres of Adult Vertebrate Skeletal Muscle

KUAN WANG and RUBEN RAMIREZ-MITCHELL*

Clayton Foundation Biochemical Institute, Department of Chemistry, and *Cell Research Institute, University of Texas at Austin, Texas 78712

ABSTRACT An extensive network of transverse and longitudinal filamentous bridges was revealed when small myofibril bundles, prepared from Triton-EGTA-treated rabbit skeletal muscles, were extracted with KI to remove the majority of thin and thick filaments. Transmission and scanning electron microscopic studies of these salt-resistant cytoskeletal residues indicated: (a) small bundles of short transverse filaments connect adjacent myofibrils by forming Z to Z and M to M bridges; (b) parallel, continuous longitudinal filaments connect the peripheries of successive Z-disks and ensheath the sarcomere. These transverse and longitudinal filaments have the characteristic morphology of intermediate filaments; (c) two rings of tightly interwoven and tangled filaments, connected laterally by short filaments, encircle each Z disk. This double-ring also encircles a weblike meshwork which penetrates the sarcomeric space. From the peripheries of these rings, transverse and longitudinal intermediate filaments emerge; and (d) a massive amount of material translocated and accumulated near Z disks during KI extraction. The residues were fairly resistant to solubilization by urea and SDS, and complete dissolution was achieved only with guanidinium chloride. SDS PAGE indicated that the residues consisted mainly of titin, nebulin, and variable amounts of residual myosin and actin. Desmin represented only a few percent of total residual proteins; however, it may be a major component of the intermediate filament network. We suggest that the intermediate filament should be considered an integral sarcomeric component that may play important cytoskeletal roles in muscle structure and mechanics.

The characteristic cross-striated appearance of skeletal and cardiac muscles arises as a result of the transverse alignment of sarcomeric striations of neighboring myofibrils. The maintenance of this alignment has been attributed to the existence of filamentous bridges between Z disks and between M-lines across the fiber axis (for reviews, see references 16, 17).

Most studies have focused on the filamentous bridges that connect neighboring myofibrils—i.e., the *transverse, interfibrillar* type. However, little attention has been paid to the possible existence of *longitudinal, intrafibrillar* bridges that may connect adjacent sarcomeric structures of the same myofibril. Residual *longitudinal* filaments spanning the gaps between IZI brushes or between Z structures¹ of the same myofibril have been

repeatedly detected in myofibrils that were depleted of thin and thick filaments by selective salt extractions (e.g. 3, 9, 13, 22), but the identity of these residual filaments remains unresolved. During our investigations of titin and nebulin—a group of large, major myofibrillar proteins (32–35)—we became intrigued by these residual longitudinal filaments and considered it possible that they represent a new type of myofilament consisting of these proteins. We have, therefore, investigated the fine structure and spatial organization of these filaments in KI-extracted rabbit skeletal myofibrils. We report that the longitudinal filaments appear to be parallel, continuous, inter-

KI extracts much of the density of Z disks and causes salt-resistant non-Z proteins to translocate and to accumulate near the remains of Z disks. Therefore, the Z structure may bear only partial resemblance in structure and in composition to the Z disk of intact muscle. (K. Wang, unpublished observations).

¹ In this paper the term “Z structure” is used to refer to the dense residual structure appearing after KI extraction at the position of “Z disk” of intact muscle. This distinction is considered important because

mediate filaments that form a sleeve surrounding each myofibril and connect the peripheries of successive Z disks.

Preliminary reports of this work have appeared in several abstracts (24, 32, 33).

MATERIALS AND METHODS

Preparation of Rabbit Skeletal Myofibrils and Myofibril Bundles

The following procedures were carried out at 4°C in a cold room.

A New Zealand white rabbit (~6 mo old) was injected intravenously with a sublethal dose of nembutal (3 ml of 50 mg/ml) and then exsanguinated. Back muscle strips (1–3 mm thick) were tied to plastic rods either at resting length or after gentle stretching to 150% of resting length and then excised. They were soaked twice in a Triton X-100-containing chemical skinning solution (1% (wt/vol) Triton X-100, 0.1 M KCl, 1 mM MgCl₂, 6.67 mM potassium phosphate, 5 mM EGTA, 0.1 mM DTT, pH 7.0) for 60 min each time, then washed three times with the skinning buffer without Triton X-100 for 30 min each time. All procedures were performed in an apparatus designed according to the principle of the Bio-Rad tube gel diffusion destainer (Bio-Rad Laboratories, Richmond, CA) which circulates buffer (400 ml for 12 strips) efficiently around the firmly attached muscle strips. The skinned muscle strips were frequently used immediately for myofibril preparation or after storage at -20°C in 50% (vol/vol) glycerol in Triton X-100-free skinning buffer for no more than 2 wk. The muscle strips were chopped into 2-mm pieces with a scalpel, suspended in 10–20 ml of the 50% (vol/vol) glycerol solution, and then blended in a VirTis Model 23 blade homogenizer (VirTis Co., Inc., Gardiner, NY) at 30-s bursts for a total of 2–10 min at a medium speed setting. Fragmentation was monitored with phase contrast microscopy. For the purpose of detecting interfibrillar connections, the blending was stopped when the majority of the myofibrils were still linked together as bundles consisting of two to five myofibrils. The preparation was washed one time with five volumes of Triton X-100-free skinning solution by centrifuging at 5,000 rpm (3,000 *g*_{max}) for 5 min in a Sorvall (DuPont Instruments-Sorvall Biomedical Div., Newtown, CT) SS-34 rotor and resuspended to 2–6 mg/ml.

Preparation and Structural Studies of Potassium-Iodide-Extracted Myofibrils

LIGHT MICROSCOPY: A drop of myofibril suspension was placed between a slide and a coverslip for a few minutes and a drop of Triton X-100-free skinning solution was added to one side of the cover slip while the buffer was withdrawn with a piece of filter paper on the other side to wash away nonadherent myofibrils. Extraction with potassium iodide solution (0.6 M KI, 0.1 M Tris·Cl, 3 mM EGTA, 3 mM MgCl₂, 3 mM Na₄P₂O₇, 5 mM Na₂S₂O₃, 0.1 mM DTT, pH 7.5) was performed immediately at room temperature by the same irrigation procedure. Individual myofibrils varied in the lag period before the commencement of extraction. Once started, however, the extraction process was rapid and was completed within 5–30 s. It was essential to perform the extraction within 10 min from the onset of the attachment of myofibrils to cover slips, because myofibrils became increasingly resistant to such extraction after prolonged attachment to the glass surface (>30 min), despite the fact that nonadherent myofibrils in solution respond normally to such a treatment.

Photomicrographs were taken with a Zeiss Universal microscope and a × 100 phase-contrast lens on Kodak Pan-X film and were developed with Microdol-X.

ELECTRON MICROSCOPY: A drop of myofibril suspension on a piece of parafilm was mixed gently with an equal volume of a 2× concentrated KI solution for 2 min at room temperature. A 100-mesh grid, coated with a glow-discharged carbon film deposited on Formvar, was then inverted *on top* of the droplet. The grid was removed after 2 min, rinsed gently with a few drops of 1× KI solution, and fixed in 2% glutaraldehyde in 25 mM cacodylate, pH 7.2, for 4 min. The fixed sample was rinsed twice with 0.1 M KCl, 0.01 M Tris·maleate, 2 mM MgCl₂, 2 mM EGTA, 0.1 mM DTT, pH 6.8, stained with a drop of 0.5% uranyl acetate for 5 s, and air-dried before observing it with a JEOL 100CX transmission electron microscope operated at 80 kV. For scanning electron microscopy: small pieces of freshly cleaved mica were placed *on top* of the KI-extracted myofibril suspension, rinsed, and fixed as described above. The fixed sample was rinsed thoroughly with water, dehydrated in ethanol, transferred through an amyl acetate in ethanol series, then critical-point dried. The samples were coated with 20 Å of Au/Pd and examined with a JEOL 100CX equipped with a high resolution scanning attachment. The microscope was operated at 40 kV and calibrated for magnification with a Fullam calibration grating (463 nm/line).

SDS GEL ELECTROPHORESIS: Samples for SDS gel electrophoresis were prepared by dissolving myofibrils or KI-residue (extracted in suspension for 14

min at room temperature and pelleted by centrifuging at 15,000 rpm for 10 min in a Sorvall SS-34 rotor) in a guanidinium chloride buffer (6 M guanidinium chloride, 0.05 M Tris·Cl, 5 mM EDTA, 10 mM DTT, pH 8.0) for 30 min at room temperature. The solubilized samples were dialyzed against 7 M urea buffer (7 M urea, 0.05 M Tris·Cl, 5 mM EDTA, 0.1 M DTT, pH 8.0) to remove guanidinium chloride and then prepared for SDS gel electrophoresis by the addition of an appropriate volume of 3× SDS sample buffer (0.03 M Tris·Cl, 3 mM EDTA, 0.12 M DTT, 3% [wt/vol] SDS, 30% [vol/vol] glycerol, 30 µg/ml Pyronin Y, pH 8.0), followed by incubation at 50 °C for 15 min. Guanidinium chloride was necessary for consistent gel patterns representative of the entire sample. The KI-residue frequently aggregated, especially after vigorous stirring or centrifugation, into a gellike material that could be solubilized only partially in SDS or urea. Both titin and nebulin resisted solubilization. The KI-residue, however, was completely dissolved in guanidinium chloride and remained soluble after being dialyzed into urea solution.

SDS samples were electrophoresed on gradient polyacrylamide gels (2–12% linear gradient) and were stained with Coomassie Blue as described (27, cf. reference 5).

DESMIN PREPARATION: Chicken gizzard desmin was purified according to Huiatt et al. (14).

RESULTS

Preservation of Myofibrillar Connections

The rapid chemical skinning procedure was developed to minimize the rapid proteolysis of titin and nebulin that occurs during conventional prolonged glycerination procedures (e.g., reference 26). This was achieved by adding 5 mM EGTA to inhibit calcium-activated proteases, by using Triton X-100 to permeabilize membranes, and by processing the muscle rapidly in the cold. It was unexpectedly difficult to prepare single myofibrils from these muscle fibers. Mechanical blending yielded mostly bundles of curved myofibrils still aligned and linked together laterally at the Z lines through bridges visible in the light microscope (Figs. 1A and 2). Further blending produced many single myofibrils with abnormal patterns (variable A-band width, spoollike A bands or zig-zag shaped sarcomeres). In contrast, mild blending was sufficient to generate

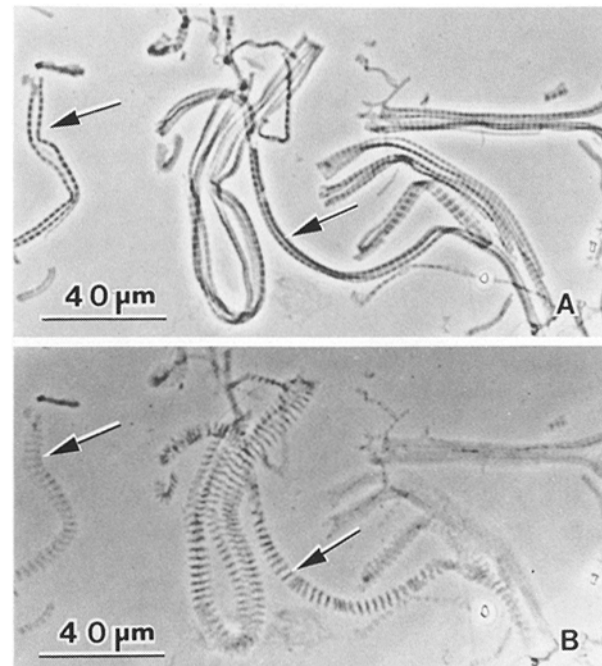


FIGURE 1 KI extraction of myofibrillar bundles. Phase contrast micrographs of myofibrils before (A) and after (B) extraction. Arrows indicate positions where Z structures have apparently filled the gaps between adjacent myofibrils. × 410.

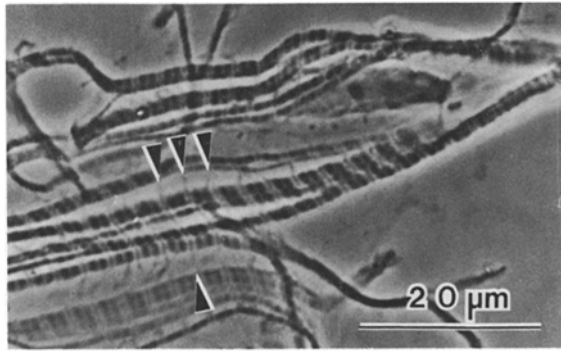


FIGURE 2 Transverse myofibrillar connections between adjacent Z disks (arrows). $\times 1,200$.

single myofibrils of normal patterns from muscle glycerinated by conventional procedures (26).

This suggests that interfibrillar connections were well-preserved in rapidly skinned fibers but were weakened in conventional glycerinated fibers. These connections must be mechanically robust, because the high shear necessary to sever the connections also produced disfigured sarcomeres.

Organization and Ultrastructure of Myofibrillar Connections

Further details of these myofibrillar connections were revealed when most of the thin and thick filaments of the myofibril were removed by extraction with KI-containing solution (13). The extraction process was rapid and efficient: within 5–30 s, A bands disappeared, residual material (containing titin and nebulin) translocated toward Z disks, and a string of thickened and darkened Z structures remained (Fig. 1*A* and *B*). The spacings between successive Z structures became increasingly irregular as the extraction progressed and, in some cases, appreciable shrinkage (20–40%) occurred along the myofibril length (data not shown). It is remarkable that Z structures remained linked and none had floated away. Furthermore, adjacent Z structures now appeared continuous because they had lengthened, filling gaps between neighboring myofibrils of a bundle. Since similar structures were observed when myofibrils were extracted in suspension, maintenance of regular spacings between Z structures did not require attachment to glass.

TEM studies of whole-mounted samples demonstrated an extensive network of filaments connecting Z structures of the same as well as adjacent myofibrils. Although individual myofibrils varied widely in the rate and the extent of extraction by KI, all sarcomeres in the same myofibril generally exhibited similar residual structures. Occasionally, one would find a single myofibril bundle (Fig. 3*A*) consisting of strings of Z structures with varying degrees of structural damage. These revealed details of myofibrillar connections and were selected for in-depth studies. A schematic representation of the organization of these filamentous bridges is presented in Fig. 7.

Interfibrillar Bridges

Transversely-aligned Z structures of adjacent myofibrils were connected by interfibrillar bridges (Fig. 3*A*), each of which consisted of 4 to 10 filaments. Most of these transverse-Z bridges (TZ) are of the same length, but longer ones occurred wherever myofibrils were farther apart, suggesting that these bridges may be extensible. Adjacent myofibrils were also con-

nected at positions corresponding roughly to M lines by bridges (TM) of one or two filaments. In residues with more extensive disintegration (as reflected by the lack of accumulated material on Z structures; see discussion below), these bridges either attached at places other than the M line position or were no longer detectable. At high magnifications, filaments of both TZ bridges and TM bridges appeared as knotty filaments that exhibited constricted (8–9 nm wide) and expanded (10–12 nm) segments alternating at ~ 25 –30-nm intervals (Fig. 3*E*). In certain regions, thin protofilaments (~ 2 nm wide) could be discerned (Fig. 3*E*). However, we were unable to determine the exact number of protofilaments. These filaments resemble intermediate filaments such as the reconstituted chicken gizzard desmin intermediate filament shown in Fig. 3*D* (also see references 14, 20, 28).

Intrafibrillar Bridges

Successive Z structures of the same myofibril were linked together by longitudinal-Z (LZ) bridges composed of ~ 50 –100 roughly parallel and equally spaced filaments. TM bridges were attached to the midpoint of one (or no more than two) of the outermost LZ filament causing the latter to bend sharply, suggesting that these longitudinal filaments may be extensible. The intrafibrillar LZ filaments also exhibited the characteristic morphology of intermediate filaments except that the knotty filament (Fig. 3*C*, and *inset*) was less frequent and many filaments had fairly uniform diameters (8–9 nm) (Fig. 3*F*). Thin protofilaments (~ 2 nm wide) could be detected in favorable cases. In one case, we observed near a Z structure a short stretch of fine axial periodicity (~ 5 nm repeat) along the laterally aligned protofilaments of several LZ filaments (Fig. 3*F*).

In summary, the ultrastructural features of both the inter- and intrafibrillar filaments, irrespective of locations, are characteristically those of intermediate filaments and are completely distinct from those of native thick or thin filaments stained identically (data not shown). Many of the thicker or curved filamentous bridges appeared to be laterally associated or twisted intermediate filament bundles. Most surface protrusions on these filaments turned out to be small membranous vesicles attached tenaciously, at irregular intervals, to these filaments.

Background Filaments

In addition to the intermediate filaments discussed above, a second type of residual filament consisting of randomly oriented, ill-defined ultra-thin filaments (2–6 nm wide) was seen on the background (Fig. 3*C*, arrowheads). The morphology of these background filaments is highly reminiscent of that of aggregated titin filaments (K. Wang and R. Ramirez-Mitchell, unpublished observations).

Z Structures

Z structures of the majority of extracted residues were heavily stained due to accumulation of translocated material (right side of Fig. 3*A*). Therefore, the filament organization near Z structures was revealed only by those samples that have little or no accumulated material (left side of Fig. 3*A*). In Fig. 3*A*, Z structures of the lower myofibril underwent a gradual morphological transition from (right to left) triangular, linear, to “Y” shaped. A similar transition was also obvious in the top myofibril, except that Z structures on the left appeared as

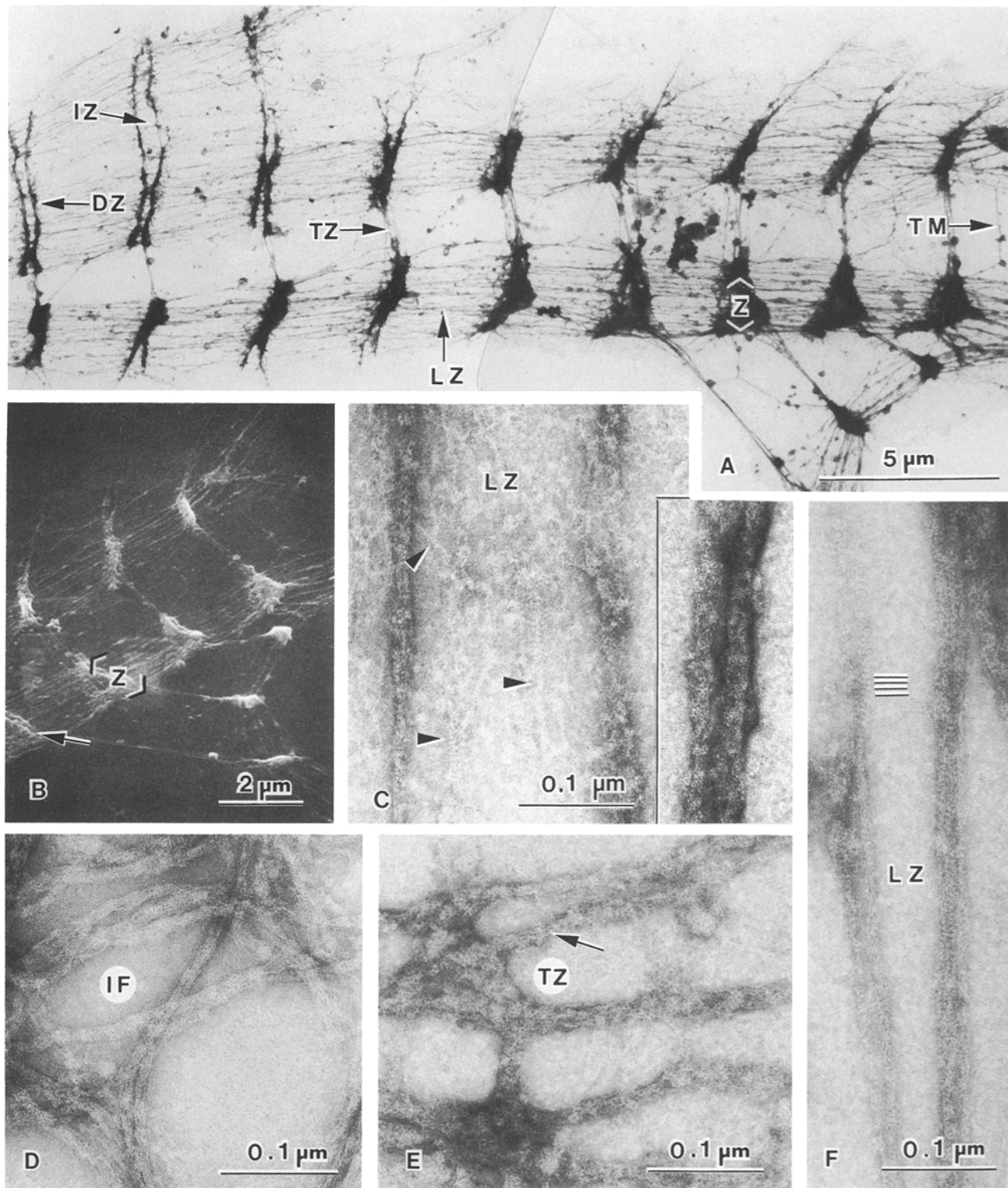


FIGURE 3 Organization and ultrastructure of myofibrillar connections. Myofibrils were extracted by KI, fixed, air dried, and stained as described in the text. (A) a bundle of highly extracted myofibrils with decreasing degrees of structural integrity (from right to left). TZ, Transverse filaments connecting Z to Z. TM, Transverse filaments connecting M to M. LZ, Longitudinal filaments connecting Z to Z. Z, Z structures. DZ, Doublet structure of highly disintegrated Z structures. IZ, Internal filaments in the gaps of doublet Z structures. $\times 6,200$. (B) Surface topology of a portion of sample (A). Sample A was coated with a gold film and examined by SEM. (Z) denotes the same Z structure in A, B, and C. Arrow indicates the two ridges of a Z structure. $\times 7,300$. (C) Fine structure of longitudinal (LZ) filaments (inset) and background ultrathin filaments (arrowheads). $\times 200,000$. (D) Fine structure of reconstituted intermediate filaments (IF) of chicken gizzard desmin. $\times 200,000$. (E) Fine structure of transverse-Z laments (TZ). Arrow indicates the region with discernible protofilaments (~ 2 nm). $\times 200,000$. (F) Fine structure of longitudinal Z filaments (LZ). Parallel lines indicate the fine axial periodicity (~ 5 nm) along the length of one of the two or three protofilaments in the bundle. $\times 200,000$.

doublets (*DZ*) with short connecting bridges (*IZ*) in between the gap (0.1–0.5 μm wide). We interpret these varied morphologies as resulting from damages to a Z structure that is constructed of two circular rings held together by connecting filaments. Breakage at one or a few points along the periphery would lead to the formation of triangular or straight Z structures; whereas additional damage on the connecting filaments would lead to “Y”-shaped or doublet morphology. The dimensions of Z structures were consistent with this interpretation: the contour length ($\sim 3 \mu\text{m}$) of the triangular and the linear Z structures corresponds to the circumference of a circular disk of $\sim 1 \mu\text{m}$ diameter. The surface topology of these Z structures was also consistent with this interpretation. SEM studies of the same field (Fig. 3A) following metal coating revealed that these Z structures represented the major bulk of the sample, while filamentous bridges appeared as very thin strands on the grid surface (Fig. 3B). Certain Z structures clearly exhibited two ridges, again supporting the idea that each Z structure may consist of two halves.

Three-dimensional Organization of Filamentous Bridges

Critical-point drying retained more faithfully certain three-dimensional features of the residues. Most Z structures appeared as fairly flattened ovals covered with a tangled mesh of filaments from which intrafibrillar filamentous bridges seemed to emerge (Fig. 4A and B). Interfibrillar bridges were difficult to discern because transversely-aligned Z structures were frequently in close contact after extraction (cf. Fig. 1B). However, outlines of individual myofibrils could frequently be demarcated by the change in packing density of LZ filaments. The LZ filaments appeared straight and taut. Thicker filaments arose from the coalescence of more than one filament emerging from the Z structure (Fig. 4D). Most LZ filaments had a mottled appearance and, in a few cases, exhibited fairly regular surface protrusions (Fig. 4D). Large membranous vesicles were frequently attached to longitudinal filaments as well as to the peripheries of Z structures.

The attachment sites of LZ filaments on the Z structure were best visualized by stereomicrographs. Most longitudinal filaments emanated from the peripheries of the flattened Z structures (Fig. 4C). Also, these filaments were continuous, which was most evident for those filaments suspended above the mica surface at the height of the Z structure. Occasionally, discontinuous filaments of variable length were seen, with one end attached to Z structures. These filaments, frequently curved and with randomly oriented unattached ends, may represent remnants of severed LZ filaments (Fig. 4C, arrow).

There were short transverse filaments that connected in a zig-zag fashion neighboring LZ filaments at the M line position (Fig. 4A and B). These frequently extended beyond the boundary of the myofibril, presumably corresponding to the TM bridges observed in Fig. 3A.

Weblike Network of Filaments in the Plane of Z Structures

Although most Z structures appeared as solid disks with no discernible internal organization, highly extracted myofibrils exhibited a branching network of filaments in the plane of the Z structure (Fig. 5). In Fig. 5A, a myofibril segment consisting of three interconnected Z structures is shown. Although portions of the dense peripheries of each of the three Z structures

were torn apart, they were still connected by transverse filaments to the bulk of the peripheries as well as by longitudinal filaments to successive Z structures. Within the boundary encircled by the peripheral fragments, there was a weblike network of filaments with openings about 0.1–0.3 μm in size. This internal meshwork is illustrated by stereomicrographs of one of the Z structures (Fig. 5C) and in the schematic interpretation (Fig. 5B). It should be noted that this sample may have been derived from a relatively large ($\sim 2 \mu\text{m}$ in diameter) myofibril, and that the peripheral fragments have been displaced radially outward after KI-extraction. The short connecting filaments seen in the gaps of doublet Z structures (Fig. 3A, *DZ*) may be the same or be derived from this internal weblike network. The fine structure of these internal filaments remains to be established.

Protein Composition of Residual Structures

KI extraction removed 80–90% of myosin, actin, and associated proteins, leaving behind titin and nebulin as the major components, and myosin heavy chain, α -actinin and actin as the minor components of the residue (Fig. 6). A 55-kdalton band co-migrating with chicken gizzard desmin was barely visible in intact myofibrils (Fig. 6A) and was enriched to a few percent by weight in the KI-residue (Fig. 6B).

DISCUSSION

Characteristic features of the filamentous network are summarized in a composite diagram in Fig. 7. Although we have depicted all of the filaments as equivalent morphologically, only transverse-Z (*TZ*), transverse-M (*TM*), and longitudinal-Z (*LZ*) filaments have been identified as intermediate-type by high resolution EM. Filaments of the dense peripheral rings and the internal meshwork of Z structures have similar morphology at low resolution, but high resolution data are lacking. Furthermore, the structural model is derived from studies of extracted rabbit myofibrils. Although it is reasonable to expect that a similar network of filaments exists in intact muscle, the detailed organization of filaments in intact muscle may deviate from this model because the extent of breakage and distortion during muscle fiber and myofibril preparation remains unknown. It also remains to be established how widely distributed such a network is in other striated muscle tissues and in other species. In a preliminary survey, we have found similar cytoskeletal networks in chicken and mouse skeletal muscles as well. It appears probable that this network may be a general feature of vertebrate striated muscles.

Transverse bridges

Filamentous bridges corresponding to TZ and TM filaments have been reported repeatedly in intact striated muscles (see reviews: 16, 17). The detection of these interfibrillar bridges was difficult unless muscles were pretreated with hypotonic solutions to increase the separation between adjacent myofibrils (12). The short connecting filaments encircling the M-line (Fig. 4B), however, have not been reported in intact muscle. Recent immunoelectron microscopic localization studies demonstrated that desmin is a component of the TZ bridges (25; see Note Added in Proof).

It has been proposed that the presence of these interfibrillar bridges is responsible for the transverse stability of the cross-striated pattern during muscle contraction and relaxation cycles (16, 17).

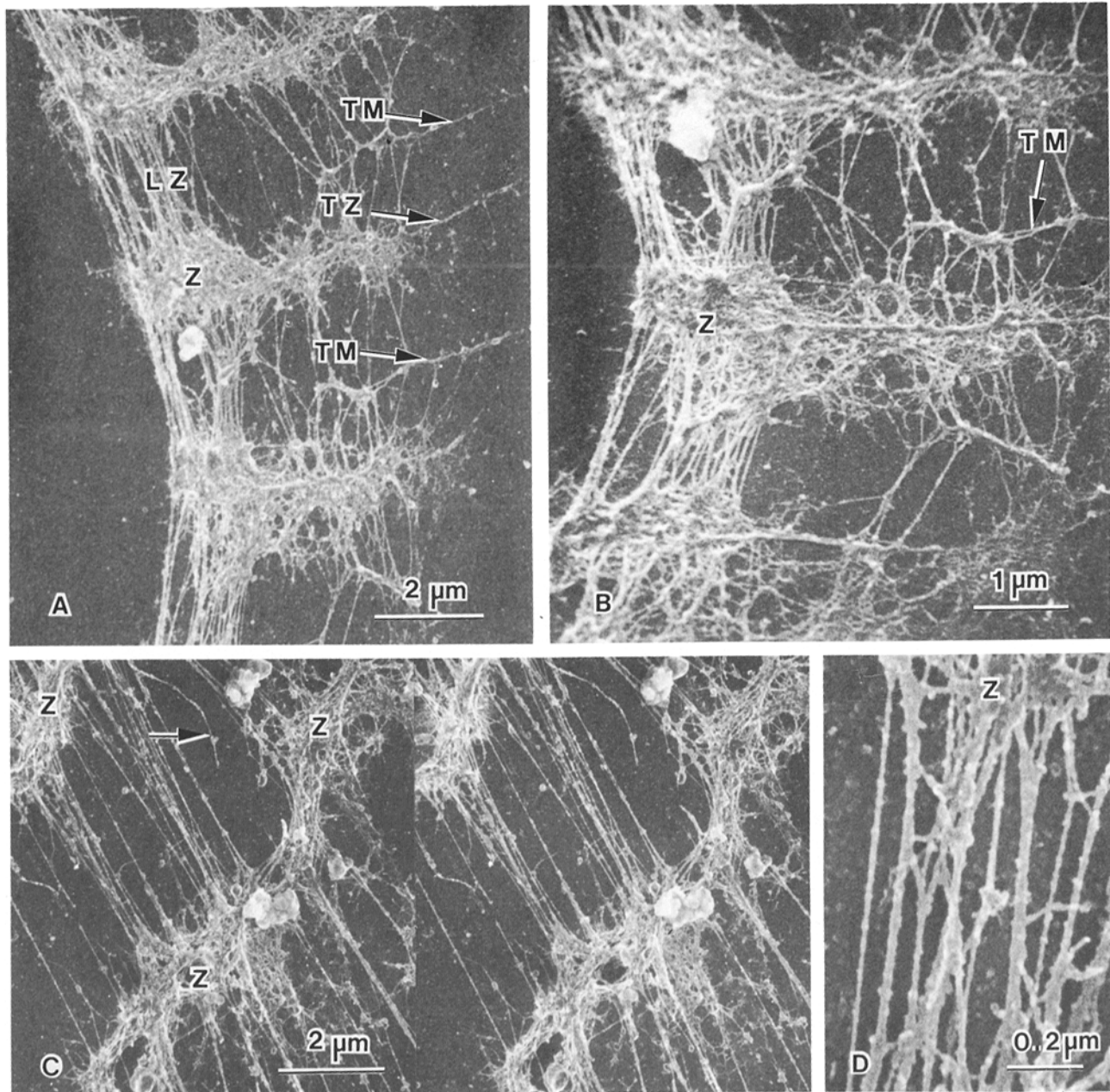


FIGURE 4 Three dimensional organization of filamentous bridges. Myofibrils were extracted by KI, fixed, critical-point-dried, and processed for SEM. (A) General morphology of filamentous bridges derived from two adjacent myofibrils. (See Fig. 3 A for abbreviations.) Note the short filaments which connect longitudinal filaments at the M position. $\times 8,400$. (B) The same as in A but at a higher magnification and a different specimen tilt. (Compare the relative positions of the globular membranous vesicle near a Z structure.) $\times 15,000$. (C) A stereopair demonstrating the peripheral location of longitudinal filaments (arrow indicates a broken filament). $\times 8,400$. (D) Fine morphology of longitudinal filaments. Note the irregular surface protrusions along the length of the filament. $\times 56,000$.

Longitudinal Bridges

Sarcomere-associated longitudinal intermediate filaments have not been observed in intact muscle. Perhaps their sparsity, their peri-sarcomeric location, their longitudinal orientation and their proximity to sarcoplasmic reticulum made them difficult to detect in thin sections (1). These filaments, however, have been reported by several workers in studies of extracted myofibrils (e.g. 3, 9, 13, 22). We consider it highly unlikely that these filaments represent translocated or residual thin and thick filaments judging from their distinct intermediate filament morphology, their continuity, their peri-sarcomeric location, and the lack of decoration by heavy meromyosin (unpublished

result). Similarly, we do not favor the proposal that these longitudinal filaments represent a new type of salt-insoluble, continuous myofilament postulated to connect successive Z disks, or the ends of thin or thick filaments to each other from *within* the sarcomere (i.e. the "third" myofilament).² Our inter-

² As will be reported in detail elsewhere, KI extraction caused the bulk of titin and nebulin, the likely components of the third myofilaments, to translocate and to accumulate on either side of Z structures. In other words, the putative third filaments have been dislodged and deranged by KI extraction, and some remnants appeared as ill-defined, ultrathin filaments on the background between Z structures (Fig. 3 C). The only residual filaments that remain connected to successive Z structures are

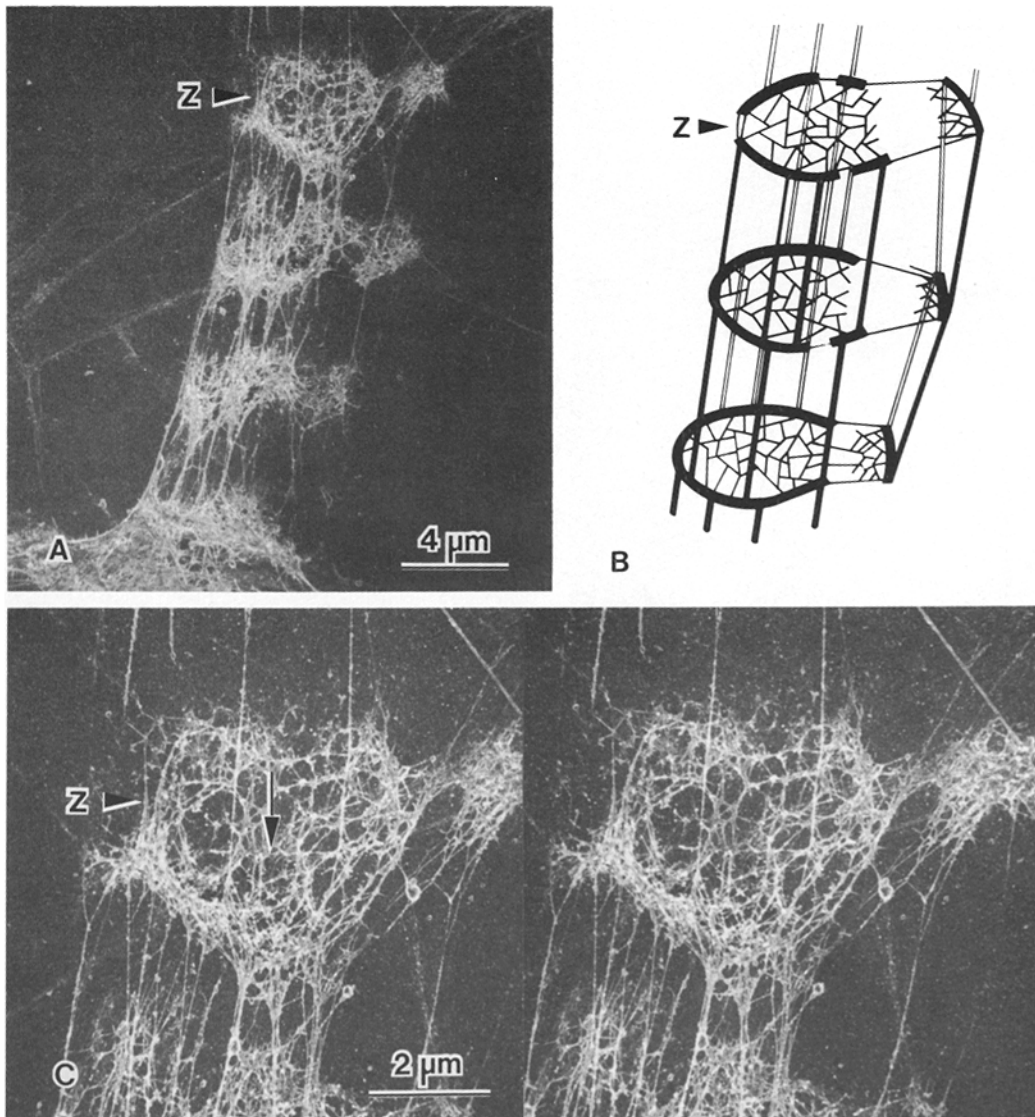


FIGURE 5 Weblike network of filaments in the plane of Z structures. Samples prepared as in Fig. 4. (A) An end-on view of three highly extracted and somewhat fragmented Z structures. Note the branching network of filaments within the plane of each Z structure. $\times 3,600$. (B) A schematic interpretation of A. Longitudinal filaments connect the peripheral fragments of each Z structure. (C) A stereopair of the top Z structure. Arrow indicates the internal network. $\times 8,300$.

pretation that these residual longitudinal filaments are intermediate filaments is consistent with a similar conclusion reached independently by Price and Sanger (22, 23) that the filaments that they observed in the gap regions of successive IZI bridges of negatively stained, A band-extracted chicken myofibrils were intermediate filaments.

The molecular composition and function of these continuous intermediate filaments remain to be elucidated. It is conceivable that they attach to and anchor sarcoplasmic reticulum and/or T-tubules which ensheath the myofibril. It is also possible that they may have a mechanical role in limiting extreme length changes of sarcomeres, e.g., by exerting a restoring force to aid overstretched or supercontracted sarcomeres to return to normal length. Such a mechanical role would require that the filaments be elastic.

the longitudinal intermediate filaments identified as LZ in Fig. 7. Failure to recognize the translocation of titin and nebulin during extraction has resulted in the misidentification of these intermediate filaments as the "third" myofibril (3, 4, 9, 19).

Z Structure Filaments

The identity of Z structure filaments remains to be established. Since a number of intermediate filament proteins such as desmin, vimentin, synemin were localized by immunofluorescence to a peripheral ring in the plane of the Z structure of KI-extracted chicken muscle (8, 9, 10, 17) and short, radially oriented intermediate filaments were seen connecting Z disks to nearby membranes in intact muscles (12, 15, 21), it is likely that at least part of the filaments in the peripheral ring, and possibly the internal meshwork, are also intermediate filaments.

In intact muscles, it is conceivable the double peripheral rings may encircle, or even penetrate the sarcomere near the Z disk region. The exact location, however, remains unclear. Because the distance between the ridges of the double rings increased from 0.1 to 0.5 μm as Z structures became progressively disintegrated (Fig. 3A), it is conceivable that the two rings may be in very close contact (with very short or no connecting filaments) and encircle the Z disk proper (as in

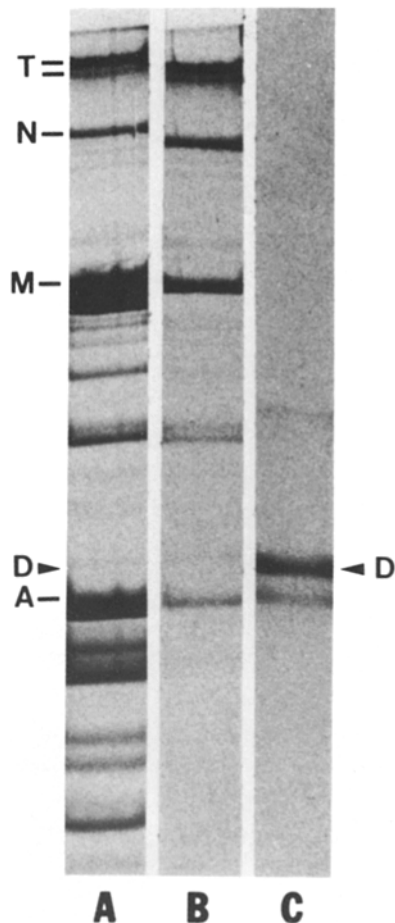


FIGURE 6 Protein composition of residual structures. A 2–12% gradient polyacrylamide gel pattern of (A) myofibrils, (B) KI residue, and (C) a mixture of desmin and actin. *T*, titin; *N*, nebulin; *M*, myosin; *D*, desmin; and *A*, actin. Note that desmin (arrowhead) comigrates with a very minor band in KI residues.

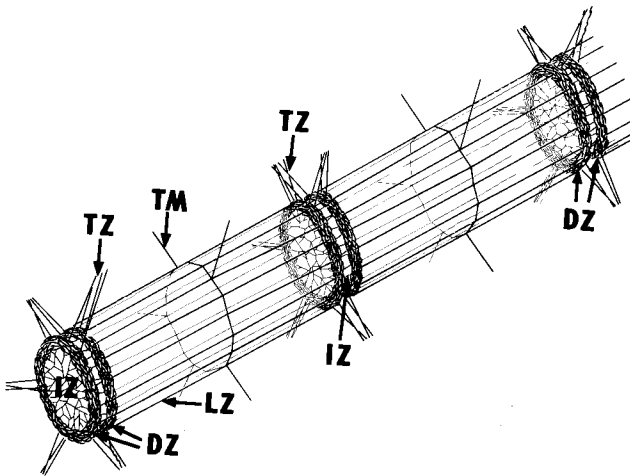


FIGURE 7 A highly schematic diagram of sarcomere-associated cytoskeletal network. Cytoskeletal filaments associated with two adjacent sarcomeres are depicted. This diagram emphasizes only the gross organizational principles of these filaments and is not drawn to scale. The disposition of cytoskeletal filaments relative to other sarcomere elements is discussed in the text. (See Fig. 3 A for abbreviations.)

Figs. 4 and 5). However, it is also possible that the double rings are situated at the positions of the pair of N_1 lines which flank the two sides of each Z disk and are 0.3–0.4 μm apart (7). The latter interpretation is attractive because internal meshwork filaments would be placed outside of the Z disk in the vicinity of N_1 lines where the spacings of the thin filament lattice would be adequate for the penetration of 10-nm wide filaments. Although the idea that intermediate filaments may penetrate into the thin filament lattice appears novel, that this could indeed happen has already been demonstrated in cardiac muscle (reference 6, plate 3). If this is also true of skeletal muscle, then each Z disk may be surrounded on *all* sides by intermediate filaments.

The double-ring structure may have implications in understanding the phenomena of Z disk splitting that was observed in certain atrophic muscle fibers, or in developing muscle where Z disks divide and move apart during the elongation of myofibrils (see reference 30 for references). These phenomena would be easier to explain if Z disks were constructed out of two halves fused together by linkages which withstand normal physiological conditions but break apart under adverse stress (see reference 30 for a detailed discussion). Our results suggest that the double ring may be involved in the construction and/or maintenance of the proposed two half Z disks.

These filamentous rings that encircle the Z disk, as well as the short connecting filaments that link LZ filaments at M line positions, may be involved in regulating the lattice spacings of thin and thick filaments; e.g., by restricting excessive lattice expansion during muscle supercontraction (29).

Protein Composition of Residual Structures

Our gel analysis indicated that titin and nebulin are consistently the major components of the residues (the relative amount of residual myosin and actin varied from preparation to preparation) and that desmin was at most a minor component. This was somewhat unexpected in view of a report which identified desmin as a major component of the KI residue of chicken myofibrils (9). This discrepancy can be explained, at least in part, by our observation that a major portion of the residue mass represented by titin and nebulin became refractory to solubilization by SDS and urea (see Materials and Methods). Therefore, the use of such ineffective denaturants led to an overestimation of the relative amount of preferentially solubilized components (9). Complete dissolution was achieved only by the use of guanidinium chloride. It is unlikely that the discrepancy is caused by proteolysis of desmin in our samples, because identical results were obtained when protease inhibitors such as phenylmethylsulfonylfluoride or leupeptin were included in various buffers. Furthermore, with our rapid skinning procedure, the extremely protease-prone titin and nebulin remained intact. Nor is it likely due to species differences, since we have obtained similar results with chicken myofibrils (unpublished result), and a recent report on porcine skeletal muscle desmin agrees with our results (25).

The fact that desmin represented only a minor component of the residue should not be interpreted to mean that it may not be a major component of the filament. This is because the photogenic filaments represent only a small portion of the residue volume (and therefore mass), as demonstrated by the scanning micrography of Fig. 3 B. The voluminous Z structures, containing translocated myofibrillar proteins, account for the majority of residue mass. It is thus possible that desmin may indeed be a major subunit of at least some of the filaments.

The question of molecular composition of these filaments should be considered open, however, in view of the well-known chemical heterogeneity of intermediate filaments (17, 28). The potential complexity in composition is already reflected by the fact that, in addition to desmin, proteins of 58 kdaltons (vimentin) (10), 68 kdaltons (31), 95 kdaltons (18), 210 kdaltons (18), 220 kdaltons (11) have been detected as intermediate-filament-associated proteins in adult skeletal muscle. Furthermore, filaments at different locations of the network may have distinct composition. This intriguing possibility is raised based on a report (18) that a 210 kdalton intermediate filament protein is localized only on M lines, whereas a 95-kdalton intermediate filament protein is localized on both M lines and as a doublet on either side of Z disks of rat skeletal myofibrils.

Although immunofluorescence studies of KI residues have indicated that myofibrillar proteins such as myosin (9), tropomyosin (9), titin (32), and nebulin (unpublished observations) occasionally appeared as longitudinal strands spanning the distance between Z structures, it is most likely that such stainings arose from extracted or dislocated proteins adhered nonspecifically to the longitudinal intermediate filaments.

Intermediate Filament Cytoskeletal Network and Muscle Tension Transmission

The intermediate filament cytoskeletal network, abundant in smooth muscle (2) and in developing striated muscles (8, 15), also appears to be intimately associated with the sarcomere of adult striated muscle. Such a cytoskeletal network has many interesting implications in muscle mechanics. It is generally assumed that muscle tension is transmitted longitudinally through the unbroken chain of serially connected sarcomeres of each myofibril to the myotendon junction. Theoretically, the intermediate filament network, by connecting all force-bearing structures, such as Z disks and M lines both longitudinally and transversely, would be capable of transmitting tension in both directions, even when certain individual sarcomeres fail to generate tension, e.g., due to nonoverlapping of thin and thick filaments or due to structural damage. Thus, this network may be an effective mechanism of bypassing defective force generators. The possible involvement of these filaments in active tension, passive tension, and elasticity of muscles remain to be explored.

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Note Added in Proof: Drs. K. T. Tokuyasu, Anne H. Dutton, and S. J. Singer (all are at University of California, San Diego) have carried out immunoelectron microscopic studies of desmin localization in

chicken skeletal and cardiac muscles. Results are detailed in manuscripts that have been submitted for publication.

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